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(12) UK Patent (19) GB (11) 2 241 329 (13) B

(54) Title of Invention

No wash immunoassay

(51) INT CL^5; G01N 33/543 33/544

(21) Application No
9100491.1

(22) Date of filing
10.01.1991

(30) Priority Data

(31) 07462827
07462828

(32) 10.01.1990

(33) US

(43) Application published
28.08.1991

(45) Patent published
09.02.1994

(52) Domestic classification
(Edition M)
G1B BCB

(56) Documents cited
EP0335244 A2
EP0125118 A
WO84/04171 A1
US4774177 A
US4642285 A

(58) Field of search

As for published application
2241329 A *viz:*
UK CL(Edition K) G1B BCB
INT CL^5 G01N
Online databases: WPI and CLAIMS
updated as appropriate

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FIG. 1

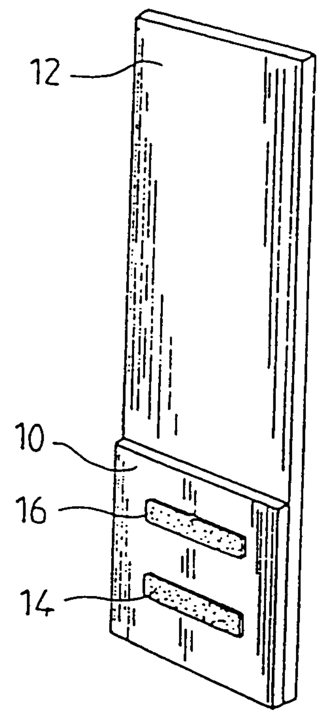
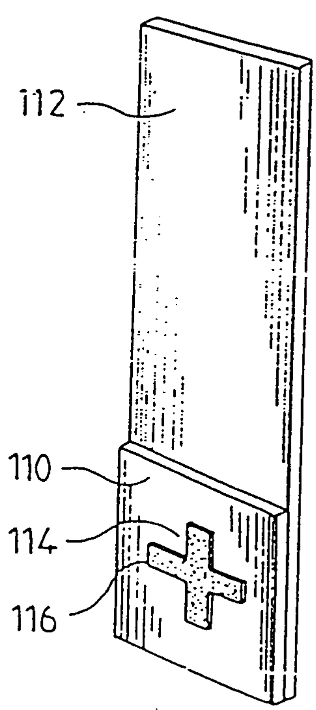


FIG. 2



NO WASH IMMUNOASSAY

This invention relates to an immunoassay device.

Various methods for detecting the presence of an analyte in a sample of biological fluid through the use of immunochemistry have been described. In the so-called "sandwich" method, for example, a target analyte such as an antigen is "sandwiched" between a labeled antibody and an antibody immobilized onto a solid support. The assay is read by observing the presence and amount of antigen-labeled antibody complex bound to the immobilized antibody. In the competition immunoassay method, antibody bound to a solid surface is contacted with a sample containing both an unknown quantity of antigen analyte and with labeled antigen of the same type. The amount of labeled antigen bound on the solid surface is then determined to provide an indirect measure of the amount of antigen analyte in the sample.

Because these and other methods discussed below can detect both antibodies and antigens, they are generally referred to as immunochemical ligand-receptor assays or simply immunoassays.

Solid phase immunoassay devices, whether of the sandwich or competition type, provide sensitive detection of an analyte in a biological fluid sample such as blood or urine. Solid phase immunoassay devices incorporate a solid support to which one member of a ligand-receptor pair, usually an antibody, antigen, or hapten, is bound. Common early forms of solid supports were plates, tubes, or beads of polystyrene which were known from the fields of radioimmunoassay and enzyme immunoassay. More recently, a number of porous materials such as nylon, nitrocellulose, cellulose acetate, glass fibers, and other porous polymers have been employed as solid supports.

A number of self-contained immunoassay kits using porous materials as solid phase carriers of immunochemical components such as antigens, haptens, or antibodies have been described. These kits are usually dipstick, flow-through, or migratory in design.

Typically, EP-A 0 125 118, disclose a sandwich type dipstick immunoassay in which immunochemical components such as antibodies are bound to a solid phase. The assay device is "dipped" for incubation into a sample suspected of containing unknown antigen analyte. Enzyme-labeled antibody is then added, either simultaneously or after an incubation period. The device is next washed and then inserted into a second solution containing a substrate for the enzyme. The enzyme-label, if present, interacts with the substrate, causing the formation of colored products which either deposit as a precipitate onto the solid phase or produce a visible color change in the substrate solution.

The requirement for washing steps and extended enzyme substrate incubation periods with dipstick immunoassay devices using enzyme labeled antibodies increases variability and thus the likelihood that minimally trained personnel and home users will obtain erroneous assay results.

The present invention comprises an immunological device, for example in the form of a dipstick, which incorporates the use of symbols in the test zone and enzyme labeled antibodies to detect analytes in a sample of biological fluid. The device thus obviates the need for the intermediate washing step associated with dipstick type enzyme labeled antibody assays. According to the present invention there is provided an immunoassay device comprising:

a base member bearing a test zone and at least one immobilized immunological component disposed in said zone in indicia-forming configuration, said immunological component capable of binding enzyme-labeled antibody through a

target analyte to form a sandwich complex on said zone which complex produces upon contact with a substrate for said enzyme a visually discernable difference between said configuration and said test zone, namely a coloured deposit which is greater in said configuration than in the remainder of said test zone.

The present invention will now be illustrated, namely by way of example, with reference to the accompanying drawing in which;

Figure 1 is a perspective view of a typical dipstick device of the present invention showing assay and control indicia in the configuration of bars; and

Figure 2 is a perspective view of a further embodiment of a dipstick device according to the present invention showing assay and control indicia in the configuration of a plus (+) sign.

Referring now to Figure 1, test zone 10 is defined on base member 12. Base member 12 may be constructed from a strip of moisture impervious material which provides a rigid support for zone 10 in order to facilitate its movement between containers. Typical base materials include but are not limited to glass or any number of plastics. Zone 10 can be constructed from a variety of elements, examples of which include but are not limited to nylon, nitrocellulose, cellulose, cellulose acetate, fiberglass, polysulfone, polyvinylidene difluoride, and polyester. An immunological component is immobilized onto zone 10 in the configuration of indicia 14, here shown as a simple bar. The immunological component is operable to bind an enzyme-labeled antibody through a target analyte, thereby forming a plurality of sandwich complexes on test zone 10 along indicia 14. A second immunological component (anti-IgG) also is immobilized in the configuration of control indicia 16, again shown as a bar. Control indicia 16 acts as an internal monitor with which to gauge assay completion.

The methods by which the antibodies are labeled with enzymes are well known in the art, as in the chemistry by which these labels cause insoluble colored products to precipitate

out of solution when brought into contact with the appropriate substrate solution.

In use, test zone 10 is inserted into a container of fluid sample to which enzyme labeled anti-analyte antibody has been added. This is allowed to stand for a short time (as for example 3-4 minutes) during which time target analyte, if present, binds to the immunological component immobilized in indicia 14 and labeled antibody binds to the analyte to form a plurality of "sandwich" complexes on test zone 10 along indicia 14. A portion of the labeled antibody which does not bind to the target analyte binds to the anti-immunoglobin G immobilized in control indicia 16. The dipstick is removed from the first container and inserted without the need for washing, into a second containing enzyme substrate. Upon contact with substrate, the enzyme labels presently extending above test zone 10 act to liberate insoluble colored products which deposit along indicia 14 and control indicia 16 in higher concentrations than in non-indicia areas of test zone 10. This results (i) in both a visible colored band in indicia 14 and in control indicia 16 if analyte is present or (ii) in only a single visible colored band in control indicia 16 if no analyte is present.

In a further embodiment of the present invention the concentration of substance immobilized in control indicia 16 is calibrated so as to provide an internal gauge of the concentration of analyte in a sample. For example, substance is immobilized in indicia 16 at a given concentration and the assay is completed. If the color that appears in indicia zone 14 is darker than that which appears in control indicia 16, the sample contains target analyte at a greater concentration than the given concentration.

Alternatively, as shown in Figure 2, a vertical indicia 114 is situated perpendicular to and overlapping control in-

dicia 116 on test zone 110 in the configuration of a plus (+) sign. Operationally, the device is used in the same manner as that described for Figure 1; now however a colored plus (+) sign becomes visible on zone 110 in the presence of target analyte, whereas if no target analyte is present a colored minus (-) sign appears.

Immunological substance can be immobilized on the test zone in any configuration which is practical or easily discernable. These configurations include but are not limited to, dots, bars, letter, numbers, and plus-minus signs. The plus-minus configuration in which anti-analyte antibody or antigen specific to a target antibody is immobilized perpendicular to a horizontal control bar of anti-Immunoglobin provides simplicity in interpreting assay results for home users since the appearance of a plus (+) sign indicates a positive result and a minus (-) sign indicates a negative result.

Printing or spraying an immunological component onto the test zone in a defined configuration provides an internal color differential on the test zone of the dipstick itself so that additional standard color charts are not needed in order to interpret assay results. In addition no washing is needed before the device is brought into contact with substrate solution. The colored substances generated deposit in greater numbers along the indicia configuration than in non-indicia areas of the test zone. Therefore washing to remove enzyme labeled antibody which has not been linked to the test zone through a target analyte is not needed.

Various enzymes can be used to label the antibodies. Typical enzymes include, but are not limited to alkaline phosphatase, horseradish peroxidase, lysozyme, glucose-6-

phosphate dehydrogenase, lactate dehydrogenase, urease, glucose oxidase, β-galactosidase, and cholesterol oxidase, with alkaline phosphatase being preferred.

Normally, once an assay has been completed the color differential generated between indicia and non-indicia areas on the test zone is great enough to provide an accurate indication of the presence or absence of a given analyte in a sample. However, this color differential can be enhanced by incorporating an enzyme inhibitor into at least non-indicia areas of the test zone membrane. Substances which exhibit relatively weak inhibitory behaviour are preferred in order to avoid unwanted inhibition of those enzymes extending above the indicia areas. In those devices especially employing alkaline phosphatase labeled antibodies, typical inhibitors include, but are not limited to, amino acids and their derivatives such as cysteine, histidine, L-phenylalanine, β-phenyl-β-alanine, p-fluorophenylalanine, and tyrosine; chelating agents such as ethylenediamine tetraacetic acid; complex oxygenated anions such as citrate, borate, arsenate, phosphate, orthophosphate, carbonate, polyestradiol phosphate, pyrophosphate, and polyphloretin phosphate; or halogenated carboxylic acid derivatives such as iodobenzoate and iodoacetamide; and color generating competitive secondary substrates such as p-nitrophenyl phosphate. Of the foregoing examples p-nitrophenyl phosphate is preferred.

When a test zone membrane which has p-nitrophenyl phosphate incorporated within non-indicia areas is brought into contact with alkaline phosphatase labeled antibodies, insoluble yellow colored products are formed as phosphate ions are cleaved. These deposit on the test zone surface in non-indicia areas, while insoluble products (usually blue in color) which result from enzyme interactions involving the

primary substrate deposit along indicia areas. Blue colored products which may deposit on non-indicia areas are masked by the yellow color.

In yet another embodiment, a first polyol is included with the enzyme inhibitor on the test zone in at least the non-indicia areas. When the alkaline phosphatase is used as an enzyme label, the polyol acts to accept phosphate ions generated as a result of enzyme-substrate interactions. This feature serves two functions.

Firstly, by coating the membrane with phosphate ions the polyol renders the membrane surface hydrophilic and therefore repellant to the hydrophobic colored products which are generated as a result of enzyme substrate contact. Since the colored products are less likely to deposit on the membrane surface a greater color differential between indicia and non-indicia areas is thereby produced.

Secondly, by acting to accept the phosphates generated, the polyol increases the rate of reaction between the alkaline phosphatase and its substrate which in turn reduces the amount of time needed to incubate the dipstick in the substrate solution. Thus, more immediate assay results are possible. Examples of polyols which may be used include but are not limited to polyvinylalcohol, polyethylene glycol, sorbitol, polypropylene glycol, and carbohydrates such as dextran, methyl cellulose, milk, and corn starch. Of the foregoing examples polyvinylalcohol is preferred.

In instances where alkaline phosphatase is not used to label the antibodies, a substance which accepts by-products of the enzyme substrate reaction thereby rendering the membrane surface repellant to the deposition of insoluble colored product and/or decreases enzyme substrate incubation periods can be incorporated onto the membrane surface.

The present invention also includes a kit in which the device, for example in the form of a dipstick, is combined with a solution of enzyme labeled antibody and a second solution of enzyme substrate which is capable of forming insoluble colored products upon contact with the enzyme label. The nature of the substrate will depend upon the particular enzyme used as a label. When for example the antibodies have been labeled with alkaline phosphatase, examples of typical substrate solutions include but are not limited to Naphthol AS-MX phosphate and Fast Blue RR salt, Naphthol AS-MX phosphate and Fast Violet B Salt, Naphthol AS-GR phosphate and Fast Blue RR Salt, 5-bromo-4-chloro-3-indoxyl phosphate (BCIP), and 3-indoxyl phosphate. When horseradish peroxidase has been used as an enzyme label typical substrate solutions include, but are not limited to, 2,2'-azino-di-3-ethylbenzthiazidine sulfonate, 3,3'-diaminobenzidine, p-phenylenediamine and pyrocatechol, 3-amino-9-ethylcarbazole, and naphthol/pyronine. Examples of a typical substrate solution for a lactate dehydrogenase label include, but are not limited to, reduced nicotinamide adenine dinucleotide (NADH), and phenazine methosulphate and nitroblue or t-nitroblue tetrazolium chloride. When antibodies are labeled with lipozyme, a typical substrate solution comprises insoluble colored substances which have been encapsulated within liposomes. A typical substrate solution for β-galactosidase labeled antibodies includes, but is not limited to, 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside. A typical substrate solution for glucose oxidase labeled antibodies includes, but is not limited to, t-nitroblue tetrazolium chloride and m-phenazine methosulfonate.

In addition to enzyme substrate, the second solution also can include a second polyol which is operable to bind phosphate ions generated as the result of contact between alkaline phosphatase and substrate. The second

polyol provides an additional means with which to decrease assay incubation periods. Typical polyols used in the substrate solution include, but are not limited to, propylene glycol, ethane glycol, butane diol, 2-amino-2-methyl-1,3-propandiol and 2-amino-2-methyl-1- propanol with 2-amino-2-methyl-1-propanol being preferred.

Additionally, the antibodies can be labelled with a direct label. Since direct labels, unlike enzyme labels, produce visually discernible signals by virtue of their concentration and not by way of a chemical interaction, the incorporation of casein or bovine serum albumin in non-indicia areas of the test zone is generally all that is necessary to produce a differential between indicia and non-indicia on the test zone. In addition the dipstick need not be incubated a second time in substrate solution. Examples of direct labels include, but are not limited to metal sols, non-metal sols, dye sols, latex particles, carbon sol, and liposome contained colored bodies.

The component which is immobilized onto the test zone in an indicia forming configuration is e.g. an antibody, antigen, or hapten, although either component of any ligand receptor interaction can be employed. The antibodies used can be monoclonal or polyclonal in origin, the production methods of which are well known in the art. The antigens and haptens can be either natural or synthetic and are available from commercial sources. In addition, the immunological components can be attached to the test zone via linking reagents which have been immobilized onto the zone in the indicia forming areas. Examples of typical linking reagents include, but are not limited to, avidin, biotin, anti-fluorescein antibody, and protein A.

The assay device is used in the detection of antibodies, antigens, or haptens. Examples of antibodies which may be detected by this device include, but are not limited to, those to Lymes disease, cytomegalovirus, Epstein

Barr virus, hepatitis and Acquired Immune Deficiency Syndrome. Examples of antigens which may be detected using this device include, but are not limited to, human chorionic gonadotropin, luteinizing hormone, and α-fetoprotein. Examples of haptens which may be detected include, but are not limited to, cocaine, tetrahydrocannabinol, digoxin, theophylline, morphine and amphetamine.

Additionally the present invention can be used in genetic screening and research to detect specific gene sequences by way of immobilized complementary sequences and labeled nucleic acid probes. These methods of gene sequence detection are known in the art.

The following Examples further illustrate the present invention.

Example 1

A. Dipstick Preparation. A test zone is prepared by attaching a sample (0.5 inch) of preactivated nylon membrane (Pall Immunodyne) with a pore size of 0.45 µm to a 0.01 inch thick plastic sheet (18 cm by 9 cm) serving as the base. The membrane is sprayed with 36 µl of 3 mg/ml sheep anti-human chorionic gonadotropin antibody in phosphate buffer solution (pH 7.4) along a line approximately 7-8 mm from the bottom using a Linomat IV (Camag). The sheet is incubated in phosphate buffer solution (pH 7.4) containing 2% dried milk (Carnation) RTM and optionally 0.5% polyvinylalcohol (MW 10,000) for 30 minutes. The sheet is washed with 5% sucrose soltuion, air dried, cut into strips (0.4 to 0.7 cm) to produce a series of like dipsticks which can be stored in a desiccator at ambient temperature.

B. Enzyme-Antibody Conjugate Preparation. Alkaline phosphatase (specific activity >1400 U/mg) is coupled to monoclonal mouse anti-human chorionic gonadotropin at a 1:1 molar ratio using the one step glutaraldehyde method. The conjugate is purified on a diethylaminoethyl Sephadex RTM A50 (5 ml) column using a linear gradient (0-1 M sodium chloride) in 10 mM Tris (pH 7.4) and 5 mM magnesium chloride. Conjugate samples (3.0 ml) are collected; assayed for enzyme activity; diluted to the concentration of 1 unit/ml in 10 mM Tris (pH 7.4), 1% bovine serum albumin, 150 mM sodium chloride, and 0.1% sodium azide; and stored at 4° C.

The conjugate is aliquoted into test tubes (10 x 75 mm) and lyophilized. The tubes are stoppered and stored.

C. Substrate Preparation. Into additional test tubes (10 x 75 mm) is aliquoted 0.8 ml of 5-bromo-4-chloro-3-indoxyl phosphate-nitroblue tetrazolium substrate solution. The tubes are stored in the dark at ambient temperature.

D. <u>Assay Performance</u>. To a tube containing conjugate is added 1.0 ml of urine standard containing 100 mIU/ml of human chorionic gonadotropin. The contents of the tube are mixed and the dipstick is inserted and allowed to stand for 4 minutes. The dipstick is removed and, without washing, inserted into a tube containing substrate solution and the tube is shaken. The tube is allowed to stand for an additional 4 minutes. The dipstick is removed from the tube and observed. The entire test zone is slightly off color and there is a discernable blue horizontal line across its surface. Assay sensitivity is measured to be 12 mIU/ml human chorionic gonadotropin.

Example 2

The same procedure as Example 1(A), 1(B), 1(C), and 1(D) is followed except that in 1(D) the dipstick is initially inserted into a tube containing only urine and the conjugate; i.e., no gonadotropin. After 4 minutes the dipstick is removed, and without washing inserted into a tube of substrate solution, incubated an additional 4 minutes, and observed. The entire test zone is slightly off color but there is no discernable line across its surface.

Example 3

The same procedures as Example 1(A), 1(B), 1(C), and 1(D) are followed except that in 1(A) the membrane is sprayed with anti-mouse immunoglobin along an additional line approximately 9-11 mm from the bottom.

The entire test zone turned slightly off color and 2 discernable blue horizontal lines formed across its surface.

Example 4

The same procedure as Example 3 is followed except that the dipstick is initially inserted into a tube which contained only urine and the conjugate, i.e. no gonadotropin.

The entire test zone turned slightly off color and only one discernable blue horizontal line (control) formed across its surface.

Example 5

The same procedure as Example 3 is followed except that the membrane is sprayed with sheep anti-human chorionic gonadotropin along a vertical line perpendicular to and bisecting the line of anti-mouse IgG in order to form a plus (+) sign configuration.

The entire test zone turned slightly off color and a discernable blue plus (+) sign formed across its surface.

Example 6

The same procedure as Example 5 is followed except that the dipstick is initially inserted into a tube which contained only urine and the conjugate.

The entire test zone turned slightly off color and a discernable blue minus (-) sign formed across its surface.

Example 7

A dipstick is prepared by the same procedure as Example 1(A) except that anti-human luteinizing hormone (LH) is used in place of anti-human chorionic gonadotropin.

Four membranes (Pall Immunodyne) are line sprayed with anti-human luteinizing hormone in accordance with the method in Example 1(A). Each membrane is then soaked in a tube of urine containing human luteinizing hormone at a given concentration (40 mIU/ml, 70 mIU/ml, 120 mIU/ml, and 200 mIU/ml). At 40 mIU/ml a slight blue band appeared on the membrane, at 70 mIU/ml a blue band of moderate intensity, at 120 mIU/ml a strong blue band , and at 200 mUI/ml a very strong blue band. A standard color chart is prepared by arranging these results in color intensity progression on a solid support.

The dipstick is inserted into a tube containing a clinical sample of urine and, without washing, into a tube of substrate solution. A moderate blue line appears on the surface of the test zone. The dipstick is compared to the color chart and it is determined that the sample contains from 40-70 mIU/ml luteinizing hormone.

Example 8

A. Preparation of Device. A dipstick is prepared in accordance with the procedure in Example 1(A) except that the membrane is line sprayed 7-8 mm from the bottom with polyclonal anti-fluorescein isothiocyanate instead of anti-human chorionic gonadotropin. A control line is sprayed with a solution of avidin approximately 9-12 mm from the bottom of the membrane.

The dipstick test zone then is blocked by soaking in phosphate buffer solution (pH 7.4) containing 2% dried milk (Carnation) and 0.5% polyvinylacetate (MW 10,000) for 30 minutes. It then is rinsed with 5% sucrose solution, air dried, and stored in a desiccator at ambient temperature.

B. Preparation of Fluorescein Isothiocyanate-Alkaline Phosphatase Conjugate (>1400 U/ml). Twenty five microliters of fluorescein isothiocyanate in 0.1 M sodium borate (pH 9.3) solution (3 mg/400 µl) is added to 5 mg of alkaline phosphatase in sodium borate at a total volume of 0.5 ml. The resultant solution is allowed to stand for 3 hours at ambient temperature.

The solution is then separated on a G25 Sephadex column and eluted with 0.1 M sodium carbonate (pH 8.5). To this is added 100 ml of a solution of 5.0 mg N-hydroxysuccinimidyl biotin in 1.0 ml dimethylsulfoxide. The resultant solution is stirred for approximately 12 hours.

Free biotin is removed by separating the conjugate on a G25 Sephadex column and eluting it with a buffer containing 10 mM Tris, 1% bovine serum albumin, 150 mM sodium chloride, and 0.1% sodium azide (pH 7.4). The conjugate is then stored at 4° C.

The conjugate solution is aliquoted into test tubes (10 x 75 mm) and then lyophilized. The tubes are stoppered and stored.

C. Substrate Preparation. Into additional tubes (10 x 75 mm) is aliquoted 0.8 ml of 5-bromo-4-chloro-3-indoxyl phosphate-nitroblue tetrazolium substrate solution. The tubes are stored in the dark at ambient temperature.

D. Competition Assay Performance. To a tube of conjugate is added 1.0 ml of urine containing fluorescein isothiocyanate (11 ng/ml). This is mixed and the dipstick is inserted and allowed to stand for 3 minutes.

The dipstick is transferred into a tube containing substrate and the tube is mixed by shaking. This is allowed to stand for 2 minutes. A dark line appears on the test zone in the control area and a faint line appears in the anti-fluorescein isothiocyanate band.

Example 9

The same procedures as Example 8(A), 8(B), 8(C), and 8(D) are followed except that in 8(D) the dipstick is initially inserted into a tube containing only urine and the conjugate, i.e. no fluorescein isothiocyanate. Two very dark lines appear on the test zone.

Example 10

A. Dipstick Preparation. A test zone is prepared by attaching a sample (0.5 inch) of preactivated nylon membrane (Pall Immunodyne) with a pore size of 0.45 μm is to a 0.01 inch thick plastic sheet (18 cm by 9 cm). A band is printed onto the membrane by air-brushing 36 μl of 3 mg/ml sheep anti-human chorionic gonadotropin antibody in phosphate

buffer solution along a line approximately 4 mm from the bottom. A second control band of sheep anti-mouse antibody is printed in the same way approximately 7 mm from the bottom of the membrane. The membrane is then allowed to air dry at ambient temperature for 15 hours.

B. Membrane Blocking. A solution is prepared from 5 mM of p-nitrophenyl phosphate and 0.2% polyvinylalcohol (MW 10,000) in 20 mM diethanolamine hydrochloride buffer (pH 8.5) containing 5 mM of magnesium chloride. The membrane is soaked in this solution for 30 minutes at ambient temperature, rinsed with 5% sucrose solution, air dried, cut into strips (5 mm), and stored in a desiccator at ambient temperature.

C. Enzyme-Antibody Conjugate Preparation. Alkaline phosphatase (specific activity >1400 U/mg) is coupled to monoclonal mouse anti-human chorionic gonadotropin at a 1:1 molar ratio using a one step glutaraldehyde method. The conjugate is purified on a diethylaminoethyl Sephadex A50 (5 mL) column using a linear gradient (0-1 M sodium chloride) in 10 mM Tris (pH 7.4) and 5 mM magnesium chloride. Conjugate samples (3.0 ml) are collected; assayed for enzyme activity; diluted to 1 unit/ml in 10 mM Tris (pH 7.4), 1% bovine serum albumin, 150 mM sodium chloride, and 0.1% sodium azide; and stored at 4° C.

The conjugate is aliquoted into test tubes (10 x 75 mm) and lyophilized. The tubes are stoppered and stored.

D. Substrate Preparation. Into additional test tubes (10 x 75 mm) is aliquoted 0.8 ml of a solution containing 5-bromo-4-chloro-3-indoxyl phosphate-nitroblue tetrazolium in 2-amino-2-methyl-1-propanol. The tubes are stored in the dark at ambient temperature.

E. Assay Performance. To a tube containing conjugate is added 1.0 ml of urine standard containing 100 mIU/ml of human chorionic gonadotropin. The contents of the tube are mixed, the dipstick is inserted and allowed to stand for 4 minutes. The dipstick is removed and inserted, without washing, into a tube containing substrate solution and allowed to remain there for an additional 4 minutes. In about 30 seconds the entire test zone turned slightly off color and two dark blue lines began to appear on the test zone surface. Assay sensitivity is measured to be 12 mIU/ml.

Example 11

The same procedures as Example 10(A), 10(B), 10(C), 10(D), and 10(E) are followed except that in 10(E) the dipstick initially is inserted into a tube containing only urine and the conjugate, i.e. no gonadotropin. After 4 minutes, the dipstick is removed and, without washing inserted into a substrate tube, incubated an additional 4 minutes, and observed. After 30 seconds the entire test zone turned slightly off color but only one dark blue line began to appear on the test zone surface.

Example 12

A number of dipstick devices were prepared in accordance with Examples 10(A) and 10(B) except that in 10(B) each membrane was soaked in one of the following reagent combinations.

1. Milk (2%) in a 10 mM phosphate buffer solution (pH 7.5) of 150 mM sodium chloride and 0.1% sodium azide.

2. Milk (2%) in 10 mM triethanolamine hydrochloride buffer (pH 8.0).

3. Polyvinylalcohol (0.5%) in a 20 mM phosphate buffer solution (pH 7.5) of 150 mM sodium chloride and 0.1% sodium azide.

4. Polyvinylalcohol (0.5%) in 20 mM Tris buffer (pH 8.0).

5. Polyvinylalcohol (0.5%) in 20 mM borate buffer (pH 8.5).

6. Polyvinylalcohol (0.1%) and 0.1% milk in 20 mM carbonate buffer solution (pH 8.0).

7. Polyvinylalcohol (0.1%) in 20 mM 2-amino-2-methyl-1-propanol buffer solution (pH 8.5).

8. 5 mM p-nitrophenyl phosphate and 0.2% polyvinylalcohol in a 20mM diethanolamine hydrochloride buffer solution (pH 8.5) containing 5 mM magnesium chloride.

9. 5 mM p-nitrophenyl phosphate and 0.2% polyvinylalcohol in a 20mM diethanolamine hydrochloride buffer solution (pH 8.0).

10. Polyvinylalcohol (0.1%), Milk (0.1%), and phenylalanine (0.5%) in triethanolamine (pH 8.0).

11. Polyvinylalcohol (0.1%) and Milk (2%) in 20 mM borate buffer solution (pH 8.5).

12. Polyvinylalcohol (0.1%) and ethylene glycol (1%) in phosphate buffer solution.

13. Sorbitol (5%) and polyvinylalcohol (0.1%) in 20 mM triethanolamine (pH 8.0).

14. Corn starch (5%) and 0.1% polyethylene glycol (MW 6000).

15. Bovine serum albumin (0.1%) in Tris buffer.

16. 20 mM Tris buffer (pH 8.0).

17. 20 mM Glycine in 20 mM Tris buffer (pH 8.0).

18. Water

19. Casein (0.3%) in phosphate buffer solution.

20. No blocking solution.

The assay signal:noise ratio for the compositions are as follows (the lower the number the better the ratio).

Table 1

BLOCKING SOLUTION	BACKGROUND COLOR	SIGNAL STRENGTH	COMBINED RESULT
#1	light	weak	6
2	light	weak	6
3	moderate	moderate	6
4	light	very strong	3
5	light	very strong	3
6	light	weak	6
7	light	strong	4
8	very light	very strong	2
9	light	strong	4
10	moderate	moderate	6
11	moderate	moderate	6
12	moderate	very weak	8
13	moderate	very weak	8
14	moderate	strong	5
15	very dark	weak	9
16	very dark	weak	8
17	moderate	weak	7
18	dark	very weak	9
19	very light	strong	3
20	very dark	very weak	10

Example 13

The same procedures as Example 10(A), 10(B), 10(C), 10(D), 10(E) are followed except that in 10(A) the membrane is sprayed with the sheep anti-human chorionic gonadotropin along a vertical line perpendicular to and bisecting the

line of anti-mouse IgG in order to form a plus (+) sign configuration. In about 30 seconds the entire test zone turned slightly off color and a dark blue plus (+) sign began to appear on the test zone surface.

Example 14

The same procedure as Example 13 is followed except that the dipstick is initially inserted into a tube which contained only urine and the conjugate, i.e. no gonadotropin.

The entire test zone turned slightly off color and a very dark blue minus (-) sign formed across its surface.

Example 15

The dipstick is prepared by the same procedure as Example 10(A) except that anti-human luteinizing hormone (LH) is used in place of anti-human chorionic gonadotropin and there is no control band.

Four membranes (Pall Immunodyne) are line sprayed with anti-human luteinizing hormone in accordance with the method in Example 10(A). Each membrane is then dipped into a tube of urine containing human luteinizing hormone at a given concentration (40 mIU/ml, 70 mIU/ml, 120 mIU/ml, and 200 mIU/ml). At 40 mIU/ml a slight but very distinct blue band appeared on the membrane, at 70 mIU/ml a very distinct blue band of moderate intensity, at 120 mIU/ml a very distinct strong blue band, and at 200 mUI/ml a very distinct and very strong blue band. A standard color chart is prepared by arranging these results in order of color intensity on a solid support.

The dipstick is inserted into a tube containing a clinical sample of urine and without washing into a tube of substrate solution. A very distinct strong blue line appears on the surface of the test zone. The dipstick is compared to the color chart and it is determined that the sample contains from 120-200 mIU/ml luteinizing hormone.

Example 16

The dipstick is prepared by the same procedure as Example 10(A) except that anti-human luteinizing hormone (LH) is used in place of anti-human chorionic gonadotropin and the control band is printed with anti-mouse antibody at the signal intensity equivalent to the concentration of 40 mIU/ml of luteinizing hormone.

The dipstick is inserted into a tube containing a clinical sample of urine allowed to incubate for 4 minutes, removed, and then inserted without washing into the substrate solution. Two distinct blue lines appear on the surface of the test zone. The second line is darker than the control line indicating the presence of greater than 40 mIU/ml of luteinizing hormone in the sample.

Example 17

A. <u>Preparation of Device</u>. A dipstick is prepared in accordance with the procedure in Example 10(A) except that the membrane is line sprayed 7-8 mm from the bottom with polyclonal anti-fluorescein isothiocyanate instead of anti-human chorionic gonadotropin. The control line is sprayed with a solution of avidin approximately 9-12 mm from the bottom of the membrane.

B. <u>Preparation of Flourescein isothiocyanate-Alkaline Phophatase Conjugate (>1400 U/ml)</u>. Twenty five microliters

of fluorescein isothiocyanate in 0.10 M sodium borate (pH 9.3) solution (3 mg/400 μl) is added to 5 mg of alkaline phosphatase in sodium borate at a total volume of 0.5 ml. The resultant solution is allowed to stand for 3 hours at ambient temperature.

The solution is then separated on a G25 Sephadex column and eluted with 0.1 M sodium carbonate (pH 8.5). To this is added 100 ml of a solution of 5.0 mg N-hydroxysuccinimidyl biotin in 1.0 ml dimethylsulfoxide. The resultant solution is stirred for approximately 12 hours.

Free biotin is removed by separating the conjugate on a G25 Sephadex column and eluting it with a buffer containing 10 mM Tris, 1% bovine serum albumin, 150 mM sodium chloride, and 0.1% sodium azide (pH 7.4). The conjugate is then stored at 4° C.

The conjugate solution is aliquoted into test tubes (10 x 75 mm) and then lyophilized. The tubes are stoppered and stored.

C. <u>Substrate Preparation</u>. Into additional tubes (10 x 75 mm) is aliquoted 0.8 ml of 5-bromo-4-chloro-3-indoxyl phosphate-nitroblue tetrazolium substrate solution. The tubes are stored in the dark at ambient temperature.

D. <u>Competition Assay Performance</u>. To a tube of conjugate is added 1.0 ml of urine containing fluorescein isothiocyanate (11 ng/ml) and the dipstick. This is mixed and allowed to stand for 3 minutes.

The device is then without washing moved into a tube containing substrate and the tube is mixed by shaking. This is allowed to stand for 2 minutes. A dark line appears on

the test zone in the control area and a faint line appears in the anti-fluorescein isothiocyanate band.

Example 18

The same procedure as Example 17 is followed except that the dipstick is initially inserted into a tube containing only urine and the conjugate, i.e. no fluorescein. Two very dark lines appear on the test zone.

CLAIMS

1. An immunoassay device comprising: a base member bearing a test zone and at least one immobilized immunological component disposed in said zone in indicia-forming configuration, said immunological component being capable of binding enzyme-labeled antibody through a target analyte to form a sandwich complex on said zone which complex produces upon contact with a substrate for said enzyme a coloured deposit which is greater in said configuration than in the remainder of said test zone.

2. An assay device according to claim 1 wherein said immobilized component is an antibody, antigen or hapten.

3. An assay device according to claim 2 wherein said immobilized component is an antibody.

4. An assay device according to any one of claims 1 to 3 wherein said enzyme is alkaline phosphatase, horseradish peroxidase, lysozyme, glucose-6-phosphate dehydrogenase, lactate dehydrogenase, urease, cholesterol oxidase, β-galactosidase, or glucose oxidase.

5. An assay device according to claim 4 wherein said enzyme is alkaline phosphatase.

6. An assay device according to claim 5 which comprises a first polyol distributed throughout said test zone.

7. An assay device according to claim 6 wherein said first polyol is polyvinylalcohol, a carbohydrate, polyethylene glycol, sorbitol, polypropylene glycol, dextran, methyl cellulose, milk or corn starch.

8. An assay device according to claim 7 wherein said first polyol is polyvinylalcohol.

9. An assay device according to any one of claims 1 to 8 wherein the target analyte is an antigen, an antibody, or a hapten.

10. An assay device according to any one of claims 1 to 9 wherein said configuration is a bar, dot, number, letter or plus sign.

11. An assay device according to any one of claims 1 to 10 wherein said base member comprises plastic or glass.

12. An assay device according to any one of claims 1 to 11 wherein said test zone comprises nylon, nitrocellulose, cellulose, fiberglass, polysulfone, polyvinylidene, difluoride, or polyester.

13. An assay device according to any one of the preceding claims wherein the test zone also comprises an immunological component in indicia - forming configuration, said component being capable of binding enzyme-labeled antibody without a target analyte.

14. An assay device according to any one of the preceding claims which comprises an inhibitor to said enzyme distributed throughout said test zone.

15. An assay device according to claim 14 wherein said inhibitor is an amino acid, an amino acid derivative, a chelating agent, a complex oxygenated anion, a secondary competitive substrate or a halogenated carboxylic acid derivative.

16. An assay device according to claim 15 wherein said inhibitor is cysteine, histidine, L-phenylalanine, β-phenyl-β-alanine, p-fluorophenylalanine, tyrosine, iodobenzoate, iodoacetamide, a citrate, ethylene diamine tetraacetic acid, a borate, an arsenate, a phosphate, an orthophosphate, a carbonate, a polyestradiol phosphate, a pyrophosphate, a polyphloretin phosphate or p-nitrophenyl phosphate.

17. An assay device according to claim 16 wherein said inhibitor is p-nitrophenyl phosphate.

18. An assay device according to claim 1 substantially as described in any one of Examples 1, 3, 5, 7 or 8 to 18.

19. A kit comprising:
an assay device as claimed in any one of the preceding claims, a first solution comprising enzyme labeled antibody, and a second solution comprising a substrate capable of forming insoluble colored products upon contact with said enzyme.

20. A kit according to claim 19 wherein said enzyme is alkaline phosphatase wherein the substrate is also operable to form phosphate ions upon contact with said enzyme, and wherein the second solution comprises a second polyol operable to bind a substantial proportion of said phosphate ions.

21. A kit according to claim 20 wherein said second polyol is propylene glycol, ethane glycol, 2-amino-2-methyl-1-propanol, butane diol or 2-amino-2-methyl-1,3-propandiol.

22. A kit according to claim 21 wherein said second polyol is 2-amino-2-methyl-1-propanol.

23. An immunoassay device comprising:
a base member bearing a test zone and at least one immobilized immunological component disposed in said zone in indicia-forming configuration, said immunological component being capable of binding both target analyte and enzyme-labeled antibody, which enzyme-labeled antibody produces upon contact with a substrate for said enzyme a coloured deposit which is greater in said configuration than in the remainder of said test zone; and
an inhibitor to said enzyme distributed throughout said zone.

24 An immunoassay device comprising:
a base member bearing a test zone and at least one immobilized immunological component disposed in said zone in indicia-forming configuration, said immunological component being capable of binding direct labeled antibody through a

target analyte to form a sandwich complex on said zone which complex produces a coloured deposit which is greater in said configuration than in the remainder of test zone.

25. A kit according to claim 19 substantially as hereinbefore described.